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RAPID PUBLICATION

MULTIPLE MITOCHONDRIAL DNA DELETIONS AND PERSISTENT HYPERTHERMIA IN A PATIENT WITH BRACHMANN-DE LANGE PHENOTYPE

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In a newborn boy with characteristics of Brachmann-de Lange syndrome (BDLS) high temperatures were observed on the second day after birth and recurred 2-6 times daily during the 7 months of the patient's life. After transient hypertonia hypotonia developed. In muscle biopsy specimen taken on the 51st day of life, serious and progressive distortion of mitochondria was observed. In several mitochondria the cristae structure was broken, other mitochondria were shrunken and the damage progressed towards further deterioration in other organelles. At several points between the myofibrils amorphous material was seen possible debris of destroyed mitochondria. Most myofibrils seemed to be intact; however, in some areas myolytic signs were present. Analysis of the mitochondrial DNA (mtDNA) showed multiple deletions in skeletal and heart muscles, liver, lung and kidney. Since the mtDNA encodes several proteins of the respiratory complexes, the deleted mtDNA certainly affected the integrity of the mitochondrial oxidative phosphorylation process by synthesis of abnormal proteins. In the present case the hyperthermia may have been a result of the mtDNA damage.

KEY WORDS: Brachmann-de Lange syndrome, mitochondrial DNA deletions, hyperthermia, thermoregulation, fever.

INTRODUCTION

The Brachmann-de Lange syndrome (BDLS) is a complex clinical entity comprising grossly malformed upper limbs with craniofacial alterations [Opitz, 1985; 1994]. As recently reviewed, there is still uncertainty about the clinical signs necessary for the diagnosis and about the range of variation in individual patients [Jackson et al., 1993]. Until now, no diagnostic biochemical or molecular markers are available. Heterogenity cannot be excluded.

Mutations of the mtDNA causes different human diseases [Wallace, 1994]. The mtDNA encodes 13 peptides of the respiratory chain; thus, several mutations can affect oxidative phosphorylation. As the percentage of mutant protein molecules increases due to the presence of heteroplasmic mutant mtDNA, the activity of the respiratory enzymes decreases reaching a tolerable energy threshold below which the overall efficiency of oxidative phosphorylation is impaired. Mitochondrial oxidative phosphorylation serves three vital and interrelated functions: reoxidation of NADH and $FADH_2$, generation of ATP, and regulation of temperature by heat generation. If the coupling is loose, enormous heat production can occur [Wallace, 1994]. Here we report a fatal case of BDLS phenotype associated with multiple mitochondrial DNA deletions and continuously increased heat production.

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CLINICAL REPORT

The male infant was born from a first, uncomplicated pregnancy to non-consanguineous, apparently healthy parents. His gestational age was 35 weeks, birth weight 2340 g. The Apgar scores were 7 (5 min) and 8 (10 min). Immediately after delivery the patient required supplementary oxygen (continued for 3 days); he was pale, dyspneic, and had hypertonia. Striking malformations were recognized immediately (see below). On the second day of life fever was observed and, recurred daily (2-6 times per day). The fever ranged between 38 and 40.3 °C and could hardly be controlled throughout the patient's life. The initially increased muscle tone persisted only for a few days, later hypotonia developed which showed slow clinical progression. No serologic evidence for rubella, cytomegalovirus, Ebstein-Barr virus, toxoplasma, HIV, RSV, HSV and adenovirus was found. The results of the CT scan of the skull were normal, and the chromosomes appeared normal. The total weight gain was only 770 g, and the patient died at the age of 7 months.

Using the categories of Jackson et al. [1993], based on the analysis of 310 individuals with BDLS, the following findings were present in the patient (the numbers in parenthesis show the incidence of the manifestation reported by Jackson et al.): low posterior hair line (78%), confluent eyebrows (92%), long eyelashes (99%), broad and depressed nasal bridge (83%), anteverted nostrils (88%), prominent philtrum (94%), thin lips (94%), downtourned angles of mouth (94%), micrognathia (84%), malformed upper limbs including oligodactyly, camptodactyly, (27%) proximally placed thumbs (72%), limitation of extension at elbows (64%), small hands with short digits (93%), clinodactly of 5th fingers (74%), single transverse palmar crease (51%), and low pitched cry (74%).

METHODS

Superficial specimens were taken from the quadriceps muscle on the 51st day of life under local anesthesia. A piece of the muscle was stretched and ligated to a glass rod. The sample was dropped into 2.5% glutaraldehyde in 50 mM K phosphate buffer for 2 hours, then postfixed in 10% osmiun tetroxide, and processed for electron microscopy as described earlier [Sumegi et al., 1990]. Another piece of the muscle specimen was dropped into liquid nitrogen immediately after removal, and was used later for DNA analysis.

Total DNA was extracted from ground powdered tissues with phenol extraction [Sambrook et al., 1989]. For Southern blots the DNA was digested with *Pvu II* for 2 hours [Zeviani, 1992], the digested DNA was electrophoresed through 0.6% agarose gel and transferred to a nylon membrane with standard methods [Sambrook et al., 1989]. The probe for the Southern blot was isolated mtDNA prepared from human placenta by the method of Palva and Palva [1985]. The pure mtDNA was labeled using a chemiluminescence kit (ECL, Amersham International) according to instructions described by the manufacturer. The procedure was repeated with [32P]dATP labeling of the probe using a random prime labeling kit (Megaprime, Amersham). The deleted bands were quantified with a photodocumentation system (EuroTech, England) using the densitometry file of the analyzing software of Advanced American Biotechnology (Fullerton, CA).

The PCR amplification method was used for search of the possible mtDNA deletions in other tissues obtained at autopsy. Primers positioned at np 3,322 through 3,339 (5'CTCCTACTCCTCATTG TA3') and 4,252 through 4,236 (5'GGGGAA TGCTGGAGATT3') were designed to amplify a piece of the NADH dehydrogenase (E.C. 1.6.5.3.) gene (ND1) which normally is rarely deleted [Corral-Debrinski et al., 1991; Wallace, 1994] (930 bp product). The amplification mixture contained 50 pmol of each primer, 200 μM of dNTP mixture (Pharmacia), 2 mM $MgCl_2$ and 2 U Taq polymerase (Promega) in a standard PCR buffer (Promega) containing 50 mM KCl, 10 mM TRIS HCl (pH 9), 1% Triton X 100 in 50 μl final volume. After 5 min at 99 °C amplification was performed for 30 cycles of denaturation (1 min 94 °C), annealing (2 min 60 °C) and extension (3 min 72 °C) with a 10 min final extension (further cycles were added if the amplification was insufficient). The PCR products were subjected to 3% Nu Sieve agarose gel and the

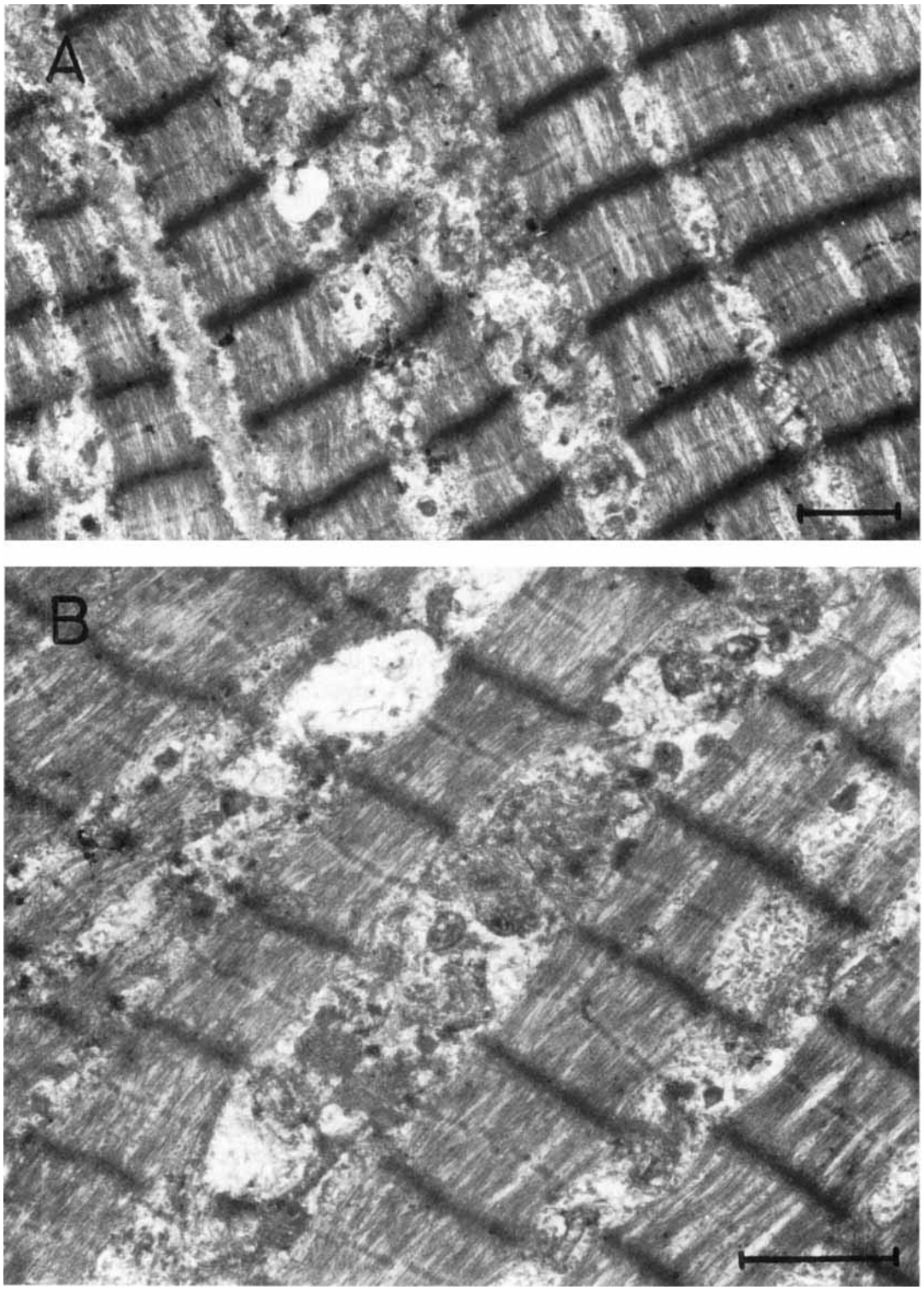

Fig 1. A. and B. : Electron micrographs of the skeletal muscle. Between myofibers amorphous, inhomogenous material is seen, the mitochondria are broken.

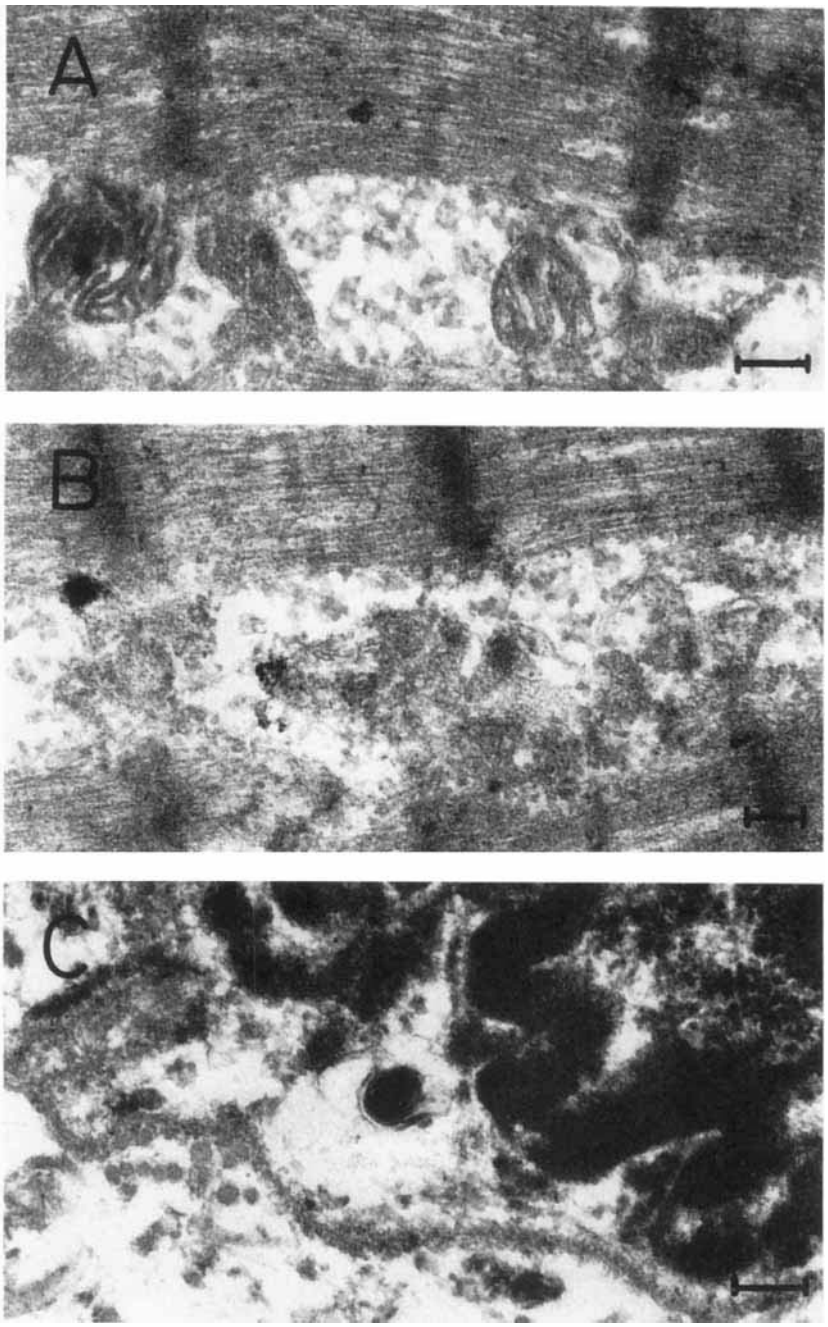

Fig 2. A.: Higher resolution electron micrographs of muscle show different degrees of mitochondrial destruction. Besides a relatively intact mitochondrion (left side) in one on the right the internal organization has disintegrated. Shrunken ghost of the mitochondria can be seen between the two less damaged mitochondria. B.: Breaks in the continuity of myofibrils are observed (lower filaments), the edge brightness of myofibrils disappearing at several points. In the space between the myofibrils inhomogene debris is observed. C.: Waste, electrondense inclusion bodies lie below the sarcoplasmatic membrane. Bars represent 250 nm.

ethidium bromide stained slabs were photographed with the above documentation system.

RESULTS

Instead of normal mitochondria amorphous, inhomogenous material was seen between the myofibers of the patient(Fig. 1 A and B). The mitochondria showed various stages of a degradation process (Fig. 2 A). In several mitochondria the organization of cristae structure had degenerated; other mitochondria were shrunken. Generally, the myofibers seemed to be normally organized. However, at several points the continuity of the fibers was disrupted, their edges showing lytic signs. In the space between the myofibrils unidentified amorphous material was seen (Fig. 2 B), probably derived from the degradation of the ghost of mitochondria and from degraded myofibrils. Electrondense material was observed predominantly under the sarcoplasmal membrane (Fig. 2 C).

Southern blot demonstrated multiple deletions of the mtDNA (Fig. 3). Instead of the normal single 16.5 kilobase digestion product, the digestion with *Pvu II* produced 7 major bands. With densitometry the following peaks could be detected (quantities are in parentheses): 16.5 kb (23%), 12.7 kb (20%), 9.20 kb (12.9%), 8.90 kb (14.1%), 8.40 kb (11.3%), 7.5 kb (8.78%) and 7.2 kb (9.92%).

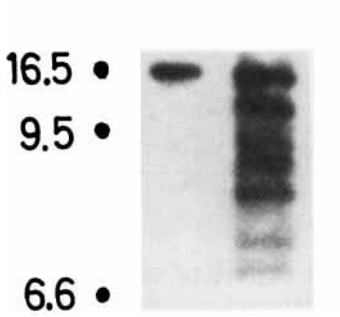

Fig. 3. Southern blot of muscle mtDNA. Left lane: single 16.5 kilobase band in the normal control; right lane: multiplex deletions of the mtDNA in the muscle of the patient.

In specimens of kidney, liver, lung and heart further smaller bands of the normally deletion-resistant ND1 gene were amplified by PCR (Fig. 4) showing, that the
mtDNA deletions were present to a different degree in the in all tissues.

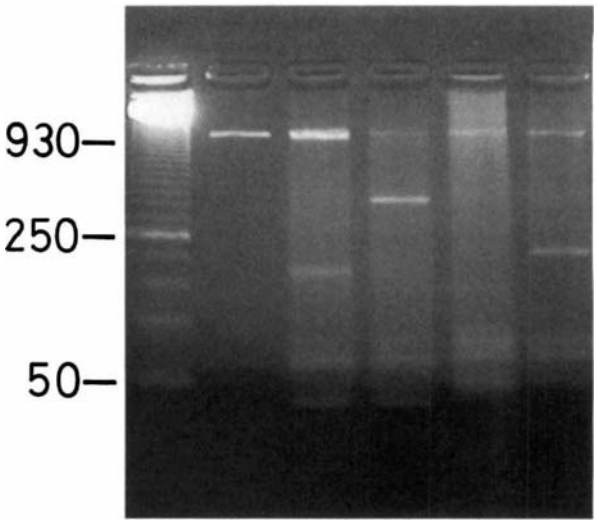

Fig. 4. PCR products of the amplification of ND1 region in different tissues obtained at autopsy. From left to right: 50 bp ladder (Pharmacia); normal blood DNA; kidney, liver, lung and heart of the patient. In addition to the normal 930 bp amplification product further 175, 80, and 70 bp fragments were found in the kidney; 400, 80 and 70 bp in the liver; 80, 70 and 50 in the lung and 300, 80, 70 and 50 bp size in the heart. The smallest band in each tissue probably corresponds to the primers.

DISCUSSION

In the syndrome first described by Brachmann [1916] and later by de Lange [1933] two major characteristic groups of malformations exist: the first affecting the limbs, the second the craniofacial region. In our case the clinical picture included abnormalities of both and allowed the diagnosis of BDLS.

In the present case muscle hypotonia and daily recurrent fever led us to search for a mitochondrial disease. In the syndrome described by Luft et al. [1962] hypermetabolism was the result of abnormal mitochondrial function and morphology; in the muscle of that patient inclusion bodies of unknown origin were observed resembling those observed in our case. As reviewed recently [Luft, 1994], several abnormalities of the mitochondria can result in the manifestations of "loosely coupled" respiration-deficient respiratory control leading to abnormal heat production. Furthermore, it became evident that the association of mitochondrial and myopathic dysfunction is common in different types of neuromuscular diseases, including those affecting the mtDNA [Editorial, 1995].

Multiple deletions of mtDNA were demonstrated in our patient; in skeletal muscle only 23% of the total mtDNA had normal molecular weight. These deletions had to affect seriously the synthesis of certain polypeptides of the respiratory chain encoded by mtDNA [Wallace, 1994]. Structure of the mitochondria was seriously affected; the morphologic findings demostrated a progressive deterioration of the organelle. As a plausible explanation, in these mitochondria loose coupling of oxidative phosphorylation could explain the abnormal heat regulation and production observed in our patient.

In BDLS no specific biochemical or molecular biology hallmarks are known, and, as it is true for several other syndromes, different genetic abnormalities can result in similar clinical phenotypes [Opitz, 1985]. The parents of the patient reported here were apparently healthy and the family history was unremarkable. Isolated new mutation in the mtDNA is not likely, since it could not explain alone the concomitant malformations. Mutation of nuclear gene(s), including those regulating the stability or the replication of the mtDNA [Zeviani et al., 1989; Zeviani, 1992], in combination with mutation of gene(s) responsible for the anatomic malformations seems to be likely. Since multiple mtDNA deletions with disturbed heat production alone or in combination with a myotonic BDLS phenotype have not been reported previously, the present condition probably represents a new syndrome.

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